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Characterization of the Heat Shock Response in *Brucella abortus* and Isolation of the Genes Encoding the GroE Heat Shock Proteins

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In an effort to define the heat shock response in the bovine intracellular pathogen *Brucella abortus*, a rough variant lacking extensive lipopolysaccharide was pulse-labeled with [^{35}S]methionine following exposure to elevated temperatures. The major heat shock proteins observed following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography migrate at 70, 62, 18, and 10 kDa. The maximum response was observed between 42 and 46°C and within 2 to 3 h of the shift in temperature and varied slightly for the different proteins. Accumulation of the 62-kDa heat shock protein (62-kDa Hsp) was observed to continue for up to 5 h following the shift in temperature. In an effort to better define the heat shock response and its potential relationship with protective immunity, genes encoding the major heat shock proteins were isolated from recombinant libraries constructed from *B. abortus* S19 and S2308 and sequenced. The 62-kDa Hsp shares more than 60% amino acid homology with members of the GroEL family and is immunoprecipitated with polyclonal antibodies to *Escherichia coli* GroEL and monoclonal antibodies to mycobacterial Hsp 65. Western blot (immunoblot) analysis with pooled sera from vaccinated and infected cattle revealed that the 62-kDa Hsp is a predominantly recognized antigen. The roles of these gene products during environmental stress and in protective immunity against brucellosis are under investigation.

Heat shock proteins are synthesized when cells are exposed to elevated temperatures or to a variety of other stresses (17). The heat shock response is highly conserved and presumably allows organisms to adapt to stressful environments. Intracellular pathogens by their very nature are exposed to a number of inhospitable environments, including extremes of pH and oxidative and nutritional stress. A stress response would presumably increase the chances of pathogen survival and dissemination; however, the members of GroEL family of heat shock proteins are conserved throughout the procaryotic kingdom and are potent immunogens which are strongly recognized by the host immune system during infection (11). As such, a potential anamnestic immune response directed against these proteins could potentially protect an infected host. GroEL also exhibits significant sequence conservation with eucaryotic heat shock proteins, which has been cited as a possible cause of autoimmune reactions (18). Clearly, further characterization of the heat shock proteins and identification of variable regions which stimulate species-specific protective immunity will help to ascertain the role that these proteins play in both survival of the pathogen and protective immunity in the host.

In experiments described below, the heat shock response of *Brucella abortus* has been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pulse-labeled whole cells. A major heat shock protein of 62 kDa (Hsp 62) is recognized by sera from cattle vaccinated with S19 or infected with field strains. The *B. abortus* 62-kDa protein was found to react with antibodies directed against the *Escherichia coli* and mycobacterial GroEL proteins in Western blot (immunoblot) and immunoprecipitation analyses. We have isolated the gene encoding the 62-kDa protein and a neighboring gene encoding the 10-kDa GroES protein. Analysis confirms their relationship with the GroE family of proteins. We have used these clones to further characterize the bovine immune response to the 62-kDa heat shock protein.

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MATERIALS AND METHODS

Chemicals and reagents. Chemicals were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, Mo.) or from the J. T. Baker Chemical Co. (Phillipsburg, N.J.). *Eco*RI, *Bam*HI, and *Hin*dIII were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). *Eco*RV was purchased from Pharmacia LKB Biotechnology (Piscataway, N.J.). [^{32}P]dATP was purchased from New England Nuclear Corporation (Boston, Mass.), and [^{35}S]methionine (Translabel) was purchased from ICN Biochemicals. Zetabind nylon membrane was obtained from Bio-Rad Laboratories (Richmond, Calif.). Sodium deoxycholate was purchased from United States Biochemical Corporation (Cleveland, Ohio). Bacteriological medium was obtained from Difco Laboratories (Detroit, Mich.). Bacteriophage lambda DNA was obtained from Sigma Chemical Co.

Bacterial strains and cultivation. *B. abortus* S19, S2308, and RB51 have been described elsewhere (3, 4) and were maintained on potato infusion agar or tryptic soy agar plates and grown to confluency for 48 to 72 h at 37°C in an atmosphere containing 5% CO_2. Minimal medium was prepared as described by Gerhardt (5) and used in pulse-labeling and pulse-chase experiments. *B. abortus* was grown in liquid culture medium, tryptic soy broth, to mid-log phase (Klett readings of 300 units with a blue filter) at 37°C and used to inoculate minimal medium at 1 to 5% (vol/vol).

Radiolabeling of *B. abortus* proteins. *B. abortus* cells were grown overnight in minimal medium to mid-log growth phase (Klett readings of 50 to 100 units in this growth medium with

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a blue filter). The cells were pelleted at 8,000 rpm in a Sorvall GS-4 rotor for 20 min and resuspended in 1/2 or 1/20 the original volume of minimal medium. Either portions of this suspension (5.0 ml or 500 μl) were heat shocked for 0 to 1 h or incubation was continued at 37°C. An equal volume of minimal medium, prewarmed to the appropriate temperature and containing [^{35}S]methionine at concentrations of 50 to 500 μCi/ml, was mixed with the cell suspension, and incubation was continued. Following incubation, the culture was adjusted to 10 mM methionine and 0.05% (wt/vol) phenol and the *B. abortus* cells were heat killed by incubation for 1 h at 65°C. The killed cells were washed three times in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.3]) and resuspended in 0.5 ml of the same solution. Triplicate 50-μl portions of each reaction mixture were precipitated with 10% (wt/vol) trichloroacetic acid and collected onto glass fiber filters, which were washed several times with 0.5% (wt/vol) trichloroacetic acid and dried under a heat lamp. [^{35}S]methionine incorporation was monitored by counting in a Beckman liquid scintillation counter (LS 3133T).

Preparation of rabbit anti-Hsp sera. Heat shock proteins were isolated from *B. abortus* RB51, which was heat shocked at 44°C for 5 h and heat killed. The cells were solubilized in 1× Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 25 mM β-mercaptoethanol, 0.001% [wt/vol] bromphenol blue), and the proteins were separated by SDS-PAGE on a 12% (wt/vol) gel. Non-heat-shock-treated cell protein was loaded on the gel as a control. The bands were visualized by staining in 5% (vol/vol) acetic acid and 7.5% (vol/vol) ethanol containing 0.25% (wt/vol) Coomassie brilliant blue. The gel was destained in the same buffer without dye. Bands corresponding to the major heat shock proteins were excised from the gel with a scalpel, and the slices were homogenized in a minimal volume of PBS by using a glass Dounce tissue homogenizer. Approximately 200 μg of each Hsp antigen was emulsified in 1.0 ml of RIBI adjuvant (RIBI Immunochem Research, Inc.), and these mixtures were inoculated into New Zealand White rabbits via intradermal injection (back), intramuscular injection (hind leg), and subcutaneous injection (neck). The rabbits were given a booster injection on day 21 with Hsp antigen in RIBI adjuvant and on day 42 with Hsp antigen without adjuvant. Blood was collected on days 20, 41, and 56. The sera were separated from the cellular fraction by centrifugation at 3,000 rpm for 15 min at 4°C in a Sorvall HB-4 rotor and stored frozen at −80°C.

Construction of genomic library, screening, subcloning, and sequencing. Preparation of the genomic library of *B. abortus* DNA was described elsewhere (3). Briefly, DNA extracted from *B. abortus* S19 and S2308 was partially digested with *Sau* 3AI, and the gel-purified fragments of 20 to 30 kbp were ligated into *Bam*HI-digested and phosphatase-treated λ2001 vector arms. The ligated DNA was packaged with commercial extracts (Stratagene) and amplified on *E. coli* P2-392. Recombinant λ2001 phage were screened following transfer to nitrocellulose filters (Schleicher & Schuell) with anti-Hsp 62 rabbit sera. Individual plaques were removed from the plates and rescreened several times until all of the plaques recovered reacted positively with the sera. The purified, recombinant phage were amplified, and progeny phage were recovered from confluently lysed plates. Phage DNA was extracted as previously described (3), digested with *Hpa*I and *Stu*I, and ligated into pBluescript KS II$^+$ plasmid cut with *Eco*RV. *E. coli* DH5α was transformed with the ligation mixture, and individual colonies were screened with anti-Hsp 62 rabbit serum. Plasmid isolated from colonies reacting with the serum contained a 2.8-kb region from *B. abortus* which was sequenced by primer extension by using the dideoxy chain termination method and unidirectional deletion mutagenesis (Erase-a-Base; Promega Biotec). The DNA sequences of *groE* genes obtained from *B. abortus* S19 and S2308 were analyzed.

Transcription-translation system and immunoprecipitation. Polypeptides encoded by recombinant plasmids were identified by using the *E. coli* S30 transcription-translation system (Promega Biotec). Radiolabeled polypeptides were separated by SDS-PAGE as described below. Following electrophoresis, the gel was impregnated with En^{3}Hance (New England Nuclear), which was precipitated with cold water, and the gel was dried under vacuum at 80°C. The radiolabeled polypeptides were visualized by autoradiography overnight at −70°C. Immunoprecipitation was performed by using polyclonal antibodies to *E. coli* GroEL, the kind gift of C. Georgopoulos (17), or to *B. abortus* Hsp 62 or monoclonal antibodies to *Mycobacterium leprae* Hsp 65, the kind gift of A. H. J. Kolk (14, 15). In all cases, protein A Sepharose was used to bind and precipitate the antigen-antibody complexes (Sigma Chemical Co.). When bovine sera were examined, goat anti-bovine antibody (Kirkegaard & Perry) was used to improve binding to protein A. Immunoprecipitated polypeptides were eluted from the protein A Sepharose in Laemmli sample buffer by boiling for 5 min and were electrophoresed and visualized as described below.

SDS-PAGE analysis. Equal amounts of protein from each of the labeled time points or different temperatures were prepared for electrophoresis. Briefly, triplicate portions of the labeled cells were analyzed for protein content by the bicinchoninic acid protein assay (Pierce Biochemicals), and equal amounts of protein were prepared for electrophoresis by heating at 95°C in 1× Laemmli sample buffer. One-dimensional gel analysis was performed on bacterial whole-cell lysates of *B. abortus* prepared as described by Sowa (12). Briefly, samples were subjected to SDS-PAGE by using the Laemmli buffer system (8) with a 4% (wt/vol) stacking gel and a 12.5% (wt/vol) separating gel. Phosphorylase *b* (molecular weight [MW], 94,000), bovine serum albumin (MW, 68,000), ovalbumin (MW, 43,000), carbonic anhydrase (MW, 30,000), soybean trypsin inhibitor (MW, 21,000), and lysozyme (MW, 14,300) from Bio-Rad Laboratories were used as molecular weight standards. Gels were stained with Coomassie brilliant blue R-250 (Sigma Chemical Co.). The stained gels were then photographed without drying or dried and exposed to X-ray film (Kodak XAR-5).

Nucleotide sequence accession number. The *B. abortus* sequence data described here have been deposited in GenBank under accession number M83930.

RESULTS

Characterization of the heat shock response in *B. abortus*. *B. abortus* is characterized by its sensitivity to heat, fastidious nutritional requirements, and low growth rate (5). With these factors in mind, the heat shock response of *B. abortus* was characterized, with special emphasis on the optimum time and duration of Hsp synthesis. *B. abortus* RB51, a stable, rough derivative of S2308, was grown overnight in minimal medium and pulse-labeled with [^{35}S]methionine at 37, 40, 42, 44, 46, and 48°C as described in the legend to Fig. 1. Protein concentrations were determined as described in Materials and Methods, and equal amounts of protein from each temperature were subjected to electrophoresis. The

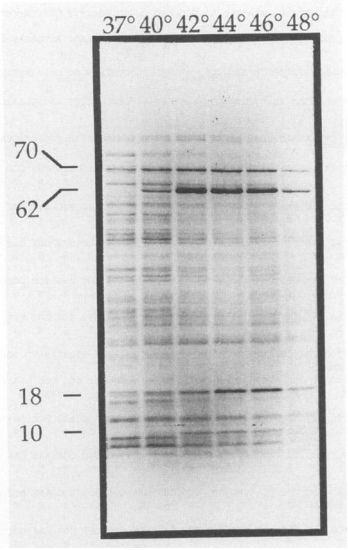

FIG. 1. Optimization of heat shock conditions in *B. abortus*. *B. abortus* RB51 was grown to mid-log phase in minimal medium, concentrated 20-fold in minimal medium, and incubated at the temperatures indicated for 30 min. The cells were then diluted with an equal volume of prewarmed minimal medium containing 200 μCi of [^{35}S]methionine per ml, and incubation was continued for an additional 60 min. The cells were heat killed as described in Materials and Methods and washed three times in PBS. The cells were resuspended in 0.5 ml of PBS, and the concentration was determined by the bicinchoninic acid assay (Pierce). Twenty micrograms of protein was heated at 95°C in Laemmli sample buffer, and the proteins were separated by SDS-PAGE. The gel was dried and exposed to XAR-5 X-ray film for 2 h. Migration of the protein standards and the molecular masses (kDa) are shown at the left.

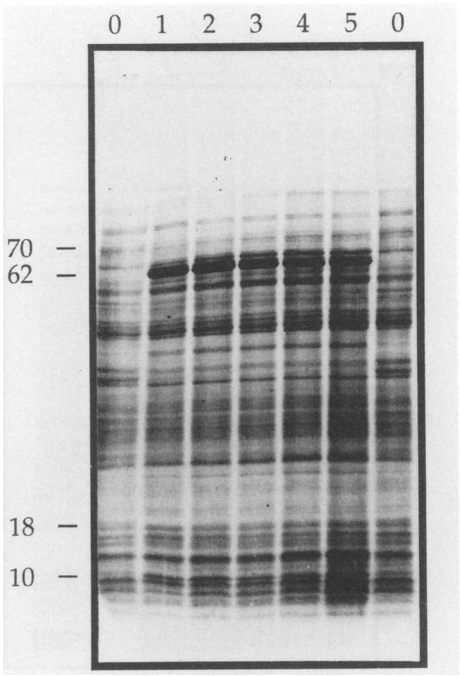

FIG. 2. Duration of heat shock protein expression. *B. abortus* RB51 was grown to mid-log phase in minimal medium, concentrated twofold, and either labeled at 37°C (0 h) or shifted to 44°C for 1 h and pulse-labeled for 1 h at hourly intervals, from 1 to 5 h following heat shock. SDS-PAGE and autoradiography were performed as described in the legend to Fig. 1.

heat shock response as characterized by one-dimensional SDS-PAGE analysis reveals a prominent increase in the synthesis of proteins having apparent molecular masses of 70, 62, 18, and 10 kDa (Fig. 2). On the basis of their apparent molecular masses, we have tentatively identified two proteins, GroEL (62 kDa) and GroES (10 kDa) (10). Although additional proteins are evident in some of the gels, their induction was not consistently observed. Induction of the synthesis of the major heat shock proteins occurs between 42 and 46°C. To characterize the duration of Hsp synthesis and its optimal time point, RB51 was pulse-labeled with [^{35}S]methionine for 1 h following a 1-, 2-, 3-, 4-, or 5-h incubation at 44°C. Protein concentrations were determined and electrophoresis was performed as described above. The results reveal the level of Hsp synthesis at various times following heat shock; maximal synthesis of Hsp 62 occurs 2 to 3 h postinduction but continues up to 5 h postinduction. This represents an unusually prolonged expression of Hsp, which may reflect the lower growth rate and possibly an environmental adaptation for this intracellular pathogen. A potential heat shock protein appearing in this gel migrates at 45 to 50 kDa but was not reproducibly observed in all experiments. The 70-kDa protein is poorly labeled under these conditions; presumably, its synthesis has drastically decreased following the 1-h preincubation at 44°C, prior to the addition of label (see below).

Western blotting (data not shown) had revealed a steady accumulation of Hsp 62 for as long as 5 h postinduction, suggesting that degradation was not a major factor in determining the protein levels during the course of labeling. Figure 3 shows the relative stabilities at 37 and 44°C of the major heat shock proteins. In this experiment, the cells were heat shocked for 30 min prior to the addition of label and subsequently labeled for 2 h at 44°C (Fig. 3, lane 2). Following the addition of methionine to a final concentration of 10 mM, the cells were chased for 3 h at either 44°C (lane 3) or at 37°C (lane 4). Lane 5 shows the absence of protein synthesis when a portion of the sample is immediately adjusted to 10 mM methionine and heated for 1 h at 65°C, indicating that protein synthesis cannot occur under these conditions. Cells which were continuously incubated at 37°C are shown in lanes 1 and 6. This experiment indicates the relative instability of the 18- and 70-kDa proteins and the stability of the 62-kDa protein. These results once again emphasize the prolonged expression and persistently elevated levels of Hsp 62 observed in this facultative intracellular pathogen.

Selection of recombinants expressing *B. abortus* Hsp 62. In order to characterize the immune response directed against the heat shock proteins of *B. abortus*, genes encoding these proteins were isolated from a genomic library described previously (3). Polyclonal rabbit antiserum directed against Hsp 62 was produced as described in Materials and Methods and used to isolate recombinants from λ2001 genomic libraries (4). Subfragments of 2.8 kbp expressing the 10- and 62-kDa proteins were cloned in pBluescript KS II$^+$ and sequenced. Two open reading frames (ORFs) of 294 (nucleotides 441 to 734) and 1,632 (nucleotides 828 to 2459) nucleotides which correspond to polypeptides of 10,381 and 57,503 Da, respectively, were found (Fig. 4). Alignment of amino acid sequences reveals a high degree of homology with GroEL analogs expressed in other organisms (Fig. 5).

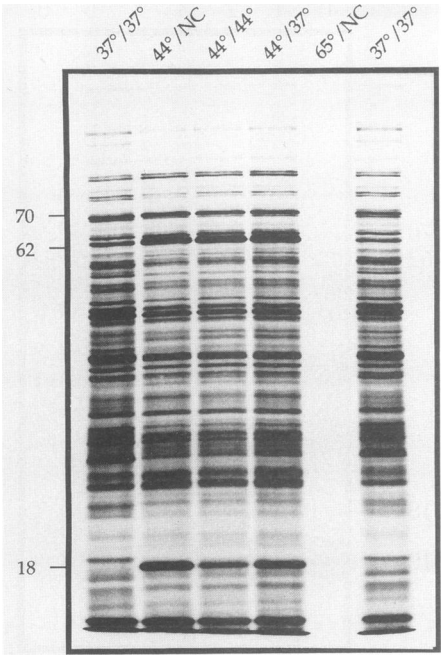

FIG. 3. Stability of *B. abortus* heat shock proteins as determined by pulse-chase labeling. *B. abortus* RB51 was pulse-labeled as described in the legend to Fig. 1, except that labeling was performed for 2 h. The label was chased during a 3-h incubation at the temperatures indicated, following the addition of unlabeled methionine to a final concentration of 100 mM. SDS-PAGE and autoradiography were performed as described in the legend to Fig. 1.

The organization of the *groES* and *groEL* genes is similar to that in *E. coli*, in which they are closely associated in a single transcription unit (7). Both polypeptides are highly expressed in *E. coli*, presumably under the regulation of their native σ^{70} promoter, and can be synthesized in a coupled transcription-translation system (Fig. 6 and 7). We have compared the putative σ^{32} promoter, σ^{70} promoter, and Shine-Dalgarno sequences with the corresponding sequence in *E. coli* (Fig. 4). The Shine-Dalgarno sequences of both ORFs occur 5 to 7 bp upstream of the ATG start codons. The σ^{32} promoter and σ^{70} promoter occur at nucleotide positions 198 to 223 (−35 region [CTTGAA]; −10 region [CCCCAG]) and 315 to 343 (−35 region [TTGACA]; −10 region [TATCTC]), respectively, and exhibit considerable sequence agreement with consensus sequences (7). In the −10 region of the σ^{32} promoter, there is a single G-for-T substitution and perfect homology at the −35 region. In the −10 region of the σ^{70} promoter, there is a CTC-for-AAT substitution and perfect homology at the −35 region. Termination of transcription appears to be under the control of a rho-independent terminator with dyad symmetry. Taken together, these results indicate that *B. abortus* GroEL is a member of the family of widely conserved heat shock proteins often referred to as common antigen (11).

Recognition of *B. abortus* heat shock proteins in vaccinated and infected cattle. When Western blots containing heat-shocked *B. abortus* RB51 are reacted with pooled bovine sera from several animals (including both protected and nonprotected) vaccinated with *B. abortus* S19, a prominent reaction with the 62-kDa protein was detected, corresponding to the GroEL homolog (data not shown). Analysis of pooled sera from prevaccinated, vaccinated, challenged, and chronically infected cattle in immunoprecipitation analysis suggested a direct correlation between exposure and humoral activity directed against Hsp 62 (Fig. 6). To determine whether the serologic response to *B. abortus* heat shock

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GATAAGCTTG ATAACGCCGT TCGGGCATGG AAACAGGCGT GGGCAAGGCC CCTTGGGTTT CCTCTACCCC AAGCGGCCCA          80

TTCTGTCACC ACGCTTCAAC AAAACGAACT GTCGCATCAT GATTTCATGA CGGATTAACC ACGGACAGAC GTTTTTGTGA         160

                                          -35        σ32        -10
AGATTAAAGC CTTTTGCGAT CATTATCGCC CACATGGCTT GAACACATG CTGCAAGCCC CAGATAGGGT AGAATGTGCA         240

                                                                                    -35
TTTCGCCACA GGCCGAAACA GGCGGAAATC CGCGTGTTCC GCGCAAAAAA CGCCAGCCTG AACGGCAATC CTCCTTGACA         320

          σ70       -10
AAAAAACATG CGGCTTCTAT CTCAAGAACC AGGTTAGCAC TCGGACGAAT AGAGTGCTAA CAGCGGGCCA TTCCGGTCCG         400

                                SD                GroES
CGTAACAGGG TTCAACGTCT ATAACACCAA GGGTTATACC ATG GCT GAT ATC AAG TTC CGC CCG CTT CAT GAC         473
                                                 M  A  D  I  K  F  R  P  L  H  D>

CGC GTC GTC GTT CGC CGC GTC GAA TCG GAA GCC AAG ACT GCC GGC GGC ATC ATC ATC CCT GAC ACT         539
 R  V  V  V  R  R  V  E  S  E  A  K  T  A  G  G  I  I  I  P  D  T>

GCC AAG GAA AAG CCG CAG GAA GGC GAA GTC GTT GCA GCC GGT GCT GGC GCT CGT GAC GAA GCT GGC         605
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 V  K  I  G  G  E  D  L  L  I  M  K  E  S  D  I  L  G  I  V  G>

TAAAAA ATTCCTTTTG CGTTGCTTCA CGCAACCAGA ATTCCATTCA CAGTACAACA TTTACAAATC TGACCGGGAT           810
 *      SD       GroEL
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 L  Q  N  A  A  S  V  A  G  L  L  I  T  T  E  A  M  I  A  E  L  P>

AAG AAG GAC GCA GCT CCG GCT GGC ATG CCT GGC GGT ATG GGT GGC ATG GGC GGC ATG GAC TTC TAA        2462
 K  K  D  A  A  P  A  G  M  P  G  G  M  G  G  M  G  G  M  D  F  *

GAAGTCCATA AAGCTCGCCC ATGAACTAAA TGCCTGCGCC GCAAAGCGGG GCGGCATGGA CTTCCAAGAA GTCCATCACC      2542

GCAGACAGGC GAGAAAGCCT GCCCCACTGC GACCAGAAAT TTAAAAACCT CCGGTTCCCG CCGGAGGTTT TTTATTCGCT      2622
                                                  <<<<<<<<        >>>>>>>
AAAATAAATA CCCATACTGG TGACATTCAC GGGTGTTACT ACCCATAATG AGCAGCAACC CCGTTTCGAT TTTAGCTCTT      2702

CCGCGTTATG TGCTGCTTAT GCAGGCTTTC CTCCCGCCCG TTGAAGTCTG CCTAACCCCG GCGAGCCCCG CTGCCGGGGT      2782
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FIG. 4. Nucleotide sequence of the *B. abortus* S19 *groE* operon. The translated amino acid sequences of GroES and GroEL ORFs are indicated below the nucleotide sequence. The putative promoter regions (−35 and −10 regions) for both σ^{70} and σ^{32} are underlined, as are the ribosomal binding sites (SD) and the dyad symmetry of the terminator stem-loop (<<< >>>). Nucleotide positions are numbered consecutively from 1 to 2775.

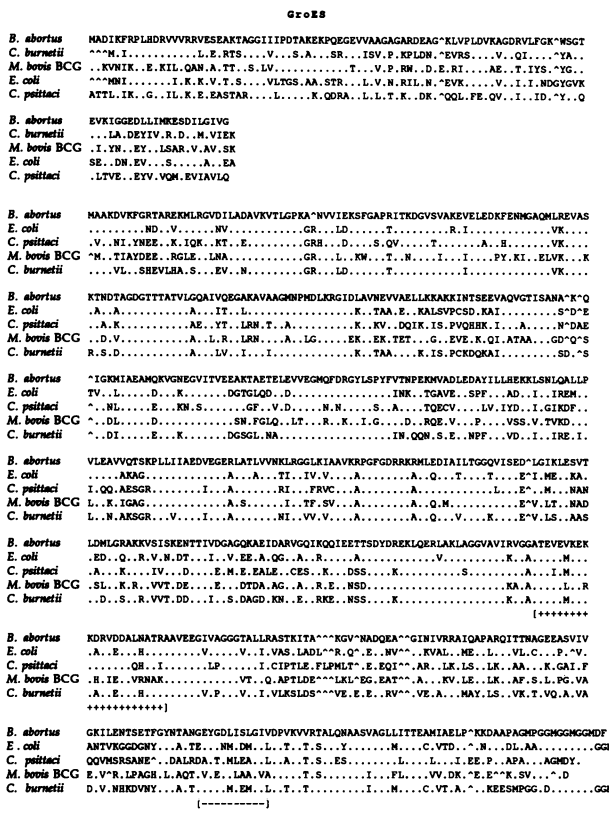

FIG. 5. Alignment of the amino acid sequences of *B. abortus* GroES and GroEL proteins with published corresponding sequences from *E. coli*, *Coxiella burnetii*, *Chlamydia psittaci*, and *Mycobacterium bovis* BCG. Identical amino acids are indicated by periods. Gaps introduced to facilitate sequence alignment are indicated by carets. + or −, reactivity of defined B-cell epitope in *B. abortus* with mycobacterial monoclonal antibodies.

proteins was specific and distinguished protected from nonprotected animals, sera from individual animals were used in a similar analysis. The results summarized in Tables 1 and 2 reveal a lack of correspondence between the humoral response to the GroEL homolog and the protection status of individual animals. In addition, the data indicate that the activity measured was present in several cattle prior to vaccination (week 0). The absence of detectable anti-lipopolysaccharide (LPS) antibodies prior to vaccination in each of these animals (1) is consistent with the absence of prior exposure to smooth *B. abortus* organisms. Since there is no known natural reservoir of rough *B. abortus* organisms, it is presumed that this activity represents an anamnestic immune response to GroEL homologs expressed by other bacterial genera.

Although sequence analysis reveals considerable variation in a number of B-cell epitopes mapped in GroEL expressed in other bacteria (Fig. 5), the serologic cross-reactivity with the *Brucella* heat shock protein was established by using anti-GroEL (*E. coli*) and anti-65-kDa protein (mycobacterial) antisera. The results shown in Fig. 7 demonstrate the ability of both *E. coli* polyclonal antibodies and mycobacterial monoclonal antibodies (F47-10 [*M. leprae*] and F67-13 [*Mycobacterium tuberculosis*]) (1a, 2, 14, 15) to interact with the *Brucella* Hsp 62. This is not unusual, since a broad cross-reactivity with these antibodies has been previously demonstrated. In contrast, no immunoprecipitation was detected

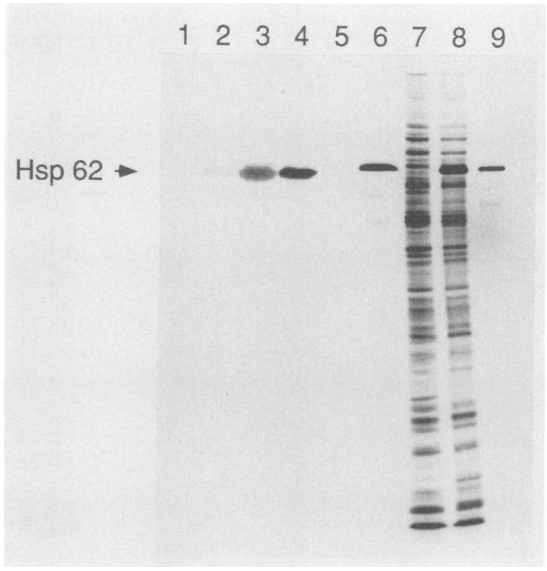

FIG. 6. Immunoprecipitation of Hsp 62 with bovine sera. *B. abortus* RB51 was labeled with [^{35}S]methionine for 1 h at 44°C as described in the legend to Fig. 1. Immunoprecipitation analysis was performed as described in Materials and Methods with pooled prevaccination (lane 1), postvaccination (lane 2), postchallenge (lane 3), and chronically infected (lane 4) bovine sera. Controls are rabbit preimmune (lane 5) and immune (lane 6) sera. Total cell proteins labeled at 37°C (lane 7) and at 44°C (lane 8) are shown. Lane 9, in vitro translation product from the cloned *B. abortus groE* locus in pBluescript KS II$^+$.

with F67-2, an antibody which has previously been demonstrated to exhibit species-restricted reactivity (15).

DISCUSSION

The most important evidence presented in this work includes (i) the demonstration of a heat shock response in *B. abortus* similar to that observed in other bacterial pathogens, but of extended duration, and (ii) the observation of a lack of correspondence between humoral recognition of the 62-kDa *B. abortus* GroEL protein in experimentally vaccinated cattle.

The first observation is not surprising; the heat shock response is highly conserved in procaryotes and eucaryotes and is expected to protect organisms, especially invasive ones, during exposure to stressful conditions. Given the facultative intracellular nature of this group of organisms, it would be interesting to examine the response of *B. abortus* to oxidative conditions or following exposure in tissue culture to macrophages. When similar experiments were performed with *Salmonella* species (another facultative intracellular bacterium), included among the proteins synthesized in response to these conditions were a number of proteins also shown to be synthesized in response to heat stress (9). Preliminary experiments in our laboratory suggest, however, that in *B. abortus*, GroEL expression is not turned on in response to oxidative conditions (2a). Since the bovine core temperature of 39°C is far from optimal in stimulating expression of heat shock proteins in vitro, one may question which factors induce expression in infected cattle. The extended duration of Hsp 62 synthesis was quite unexpected. Given the low growth rate of this organism, it is not unusual for Hsp synthesis to be longer than that observed in *E. coli*. However, this is not observed with all heat

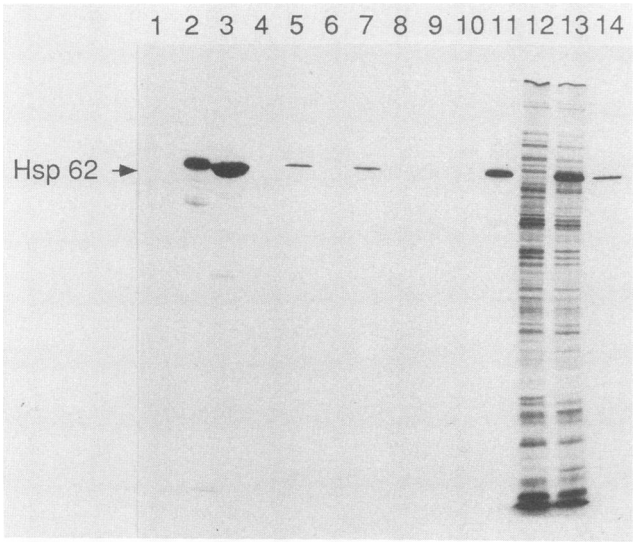

FIG. 7. Immunoprecipitation of Hsp 62 with antibodies raised against GroEL homologs. Immunoprecipitation was performed as described in Materials and Methods and the legend to Fig. 6. The following antibodies were tested for their ability to immunoprecipitate *B. abortus* GroEL synthesized in the in vitro transcription-translation system (lanes 1 to 7) or labeled in vivo at 37°C (lanes 8 to 9) or at 44°C (lanes 10 to 11): rabbit preimmune serum (lanes 1, 8, and 10); rabbit anti-*B. abortus* GroEL serum (lanes 2, 9, and 11); rabbit anti-*E. coli* GroEL (lane 3); mouse immunoglobulin G1 (IgG1)-negative control (lane 4); murine monoclonal antibody F47-10 (IgG1) (lane 5); murine monoclonal antibody F67-2 (IgG1) (lane 6); and murine monoclonal antibody F67-13 (IgG1) (lane 7). Controls include total-cell protein labeled in vivo at 37°C (lanes 12) or at 44°C (lanes 13) or synthesized in vitro (lane 14).

shock proteins. Note for example the relatively short duration of Hsp 70 synthesis. This situation is reminiscent of heat shock in *Streptomyces albus* and *Caulobacter crescentus*, two differentiating bacteria (6) which also exhibit a prolonged synthesis of GroEL and much shorter duration for the 70-kDa protein (DnaK). In those organisms, transcription of *groE* has been postulated to be under the control of a thermostable σ factor. Future research in our laboratories will address this question regarding the genus *Brucella*.

Decreased synthesis of proteins expressed under normal conditions is also often cited as a common feature of heat shock (10). In *B. abortus*, however, decreased synthesis of proteins is apparently the exception rather than the rule (Fig. 1 to 3). The continued synthesis of proteins during heat shock may correspond to the life-style of this organism, which as a facultative intracellular parasite survives and replicates under stressful conditions. Continued protein synthesis would permit continued growth during adaptation to the intraphagocytic milieu. One prominent protein whose synthesis is reduced has been identified as the *Brucella* porin of 36 kDa (Fig. 2 and data not shown). Reduction in the synthesis of this protein may be explained by the need to adapt to stressful conditions by reducing the hydrophilic permeability of the cell; however, it is expected that this would also limit the availability of nutrients. These results point out the need for continued research to better define the transcriptional activity present in these cells when subjected to stressful conditions as they are encountered in the host.

TABLE 1. GroEL antibody response in individual bovine sera for *B. abortus* S19-vaccinated cows

Cow	Degree of immunoprecipitation at wk postvaccination (date)[a]		
	0 (5 May 1988)	4 (6 June 1988)	8 (4 July 1988)
1203	++	+	+
1248	−	+	+
1254	+	+	+++
1269	+	+	+
1313	+	+	+
1375	++	++	+

[a] Examples of immunoprecipitation as shown in Fig. 6. −, no detectable immunoprecipitation; + to +++++, positive immunoprecipitation, from trace amounts to complete (see also Table 2).

TABLE 2. GroEL antibody response in individual bovine sera for unvaccinated infected cows

Cow	Degree of immunoprecipitation[a]
62	+++
166	+++
255	+
280	+
367	+++++
465	+

[a] See footnote to Table 1 for explanation of scale.

The second major observation presented in this work is the lack of correspondence between humoral immune response to GroEL and exposure status of cattle. Comparison of the predicted B-cell epitopes in *B. abortus* GroEL with those in either *E. coli* or *M. bovis* reveals homologies consistent with the antibody precipitation data (Fig. 7). Although individual infected animals with elevated antibody titers against GroEL have been identified (Tables 1 and 2), experimental animals vaccinated with S19 revealed no statistically significant increase in humoral activity directed against this protein during a period of elevated antibody titer against *B. abortus* species. In fact, several of the animals examined had greater reactivity prior to experimental exposure.

The absence of detectable change in humoral antibody titer in cattle successfully vaccinated with S19 argues against a role for GroEL in humoral immunity. This is not surprising, given the minor role predicted for humoral immunity in protection against bovine brucellosis (16). What is not clear is whether these proteins activate a protective T-cell response.

Finally, it is important to note that in many of the sera examined in this study, activity directed against the GroEL protein was observed in cattle which had never been vaccinated and, as far as can be determined, never been exposed to *B. abortus*. This was determined by standard serological methods, including the card test, rivanol, complement fixation, hemolysis-in-gel, and enzyme-linked immunosorbent assay (13). None of the animals examined had antibodies directed against the *B. abortus* LPS, a potent immunogen present on the surface of these cells. The anti-Hsp activity presumably arises because of previous exposure to other bacteria which are capable of expressing a related protein. A more careful analysis that uses oligopeptides containing epitopes specific for the *Brucella* protein will be helpful in confirming this hypothesis.

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